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(54) FABRICATION DE PROTEINES A FEUILLET PLISSE BETA ET A PROPRIETES DE LIAISON SPECIFIQUES
(54) FABRICATION OF BETA-PLEATED SHEET PROTEINS WITH SPECIFIC BINDING PROPERTIES

(57)

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(54) Titre : FABRICATION DE PROTEINES A FEUILLET PLISSE BETA ET A PROPRIETES DE LIAISON SPECIFIQUES
(54) Title: FABRICATION OF BETA-PLEATED SHEET PROTEINS WITH SPECIFIC BINDING PROPERTIES

(57) Abrégé/Abstract:
The invention relates to novel beta-pleated sheet proteins with specific binding and catalytic properties. The invention also relates to a method for producing proteins of this type.

ABSTRACT

The present invention describes novel beta-sheet proteins having specific binding properties and catalytic properties and also methods for preparing these proteins.

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Design of beta-sheet proteins with specific binding properties

The present invention relates to novel beta-sheet proteins with new or altered specific binding properties or a new or altered catalytic activity or new or altered fluorescence properties and also to methods for preparing proteins modified in such a way.

Antibodies and derivatives thereof are used in many areas of human and veterinary therapy, diagnostics and monitoring. One problem of utilizing the naturally occurring antibodies is the preparation thereof. The antibodies are still produced in an animal cell culture system, which is a very costly method. In some applications such as, for example, preparation of fusion proteins or a therapeutic use which requires rapid blood clearance and good tissue penetration, the size of naturally occurring antibody molecules represents another problem (Colcher et al., 1998). Recombinant antibody molecules such as scFvs (Bird et al., 1988), Miniantibodies (Pack and Plückthun, 1992) or bispecific antibodies (Holliger and Winter, 1993) are mainly composed of just the antigen-binding domains of the antibodies (VH and VL). Owing to their considerably reduced size, they show improved tissue penetration and are also better suited for fusions with other proteins than complete antibodies. Compared with the latter, though, recombinant antibody fragments are often more unstable, have low affinities and are difficult to prepare in recombinant form, owing to the disulphide bridges to be formed. Methods for stabilization and improved affinity of the recombinant antibody fragments include, inter alia testing various linker peptides and introduction of disulphide linkages (Glockshuber et al., 1990, Cumber et al., 1992, Brinkmann, 1997).

The sequence and length of the linker peptides can influence both the stability against proteases and the affinity of the antibody fragments (Pantoliano et al., 1991). The introduction of additional disulphide linkages into the conserved framework regions of the

variable domains can lead to increased resistance to heat (Young t al., 1995) and denaturing agents and to increased yields in heterologous expression. In general, however, many scFvs show low stability and tend to aggregate already at 37°C. The instability may also be caused by using the common Fv-fragment cloning primers which can introduce new destabilizing mutations. The antibody fragments are produced in the bacterial system mainly by exporting into the periplasmic space, and optimizations regarding the redox state and simultaneous expression of foldings helpers are possible here, too.

It is an object of the present invention to provide novel proteins having new or altered binding properties, for example antibody-like properties, but, at the same time, do not display the above-described disadvantages of complete or recombinant antibody molecules.

It is a further object of the present invention to provide proteins which display new or altered enzymic or catalytic properties.

It is another object of the present invention to generate methods for forming the abovementioned proteins.

The objects mentioned above are achieved by a protein having the features characterized in Claim 1. A method for preparing the proteins of the invention ensues from Claim 21. Preferred embodiments of the invention ensue from the subclaims and the description below.

Alteration of the surface of a beta-sheet protein generates novel binding properties previously not present in the protein. These binding properties are generated by mutagenesis of a beta-sheet region. In spite of the *de novo* binding properties, the novel beta-sheet proteins are similar to the starting proteins with respect to structure and stability. Starting proteins for designing the novel binding molecules are proteins with a predominant beta-sheet structure such as, for example, gamma-crystalline, a structural protein of the eye lens. Based on the crystal structure, regions and amino acids in the beta-sheet of the starting proteins, which are exposed on the surface and thus accessible to the solvent or possible binding partners, are selected, for example, by means of computer analyses. Using genetic engineering methods, these regions or amino acid positions are mutagenized in the gene coding for the starting protein. Thus, a multiplicity of mutated genes (bank or library) coding for the different beta-sheet proteins mutants are prepared at the DNA level. Mutants having

novel, desired binding properties are isolated with the aid of a suitable selection system such as, for example, the phage display system. In phage display, all protein mutants produced are exposed on the surface of bacteriophages (phage display library). These recombinant phages are studied with respect to their binding to the desired target molecules. Phages which expose on their surface beta-sheet mutants with specific binding to the target molecule are concentrated by repeated screening and isolated. Genes coding for binding beta-sheet mutants are obtained from the phages and expressed in a suitable expression system such as, for example, *E. coli*. Using the method described, it is surprisingly possible to prepare specifically binding proteins from beta-sheet proteins having no specific binding properties whatsoever, and mutants having the desired specificity are isolated from the library by applying a suitable screening method. Depending on the properties of the starting proteins, the beta-sheet mutants produced using the system described have advantages regarding size, stability and functionally active production in the heterologous, preferably bacterial, system, compared with, for example, antibodies and recombinant antibody fragments. These improved properties of the novel beta-sheet mutants make it possible to replace, for example, antibodies, recombinant antibody fragments of catalytic antibodies and to open up completely new application areas.

For example, the problems with antibodies, as illustrated above, can be solved according to the invention by designing proteins which have in each case specific binding properties and high stability against low pH, denaturing agents and elevated temperature, i.e. which withstand conditions under which antibodies are unstable. Generating proteins with beta-sheet structure and antibody-like binding properties, however, is only one possible field of application of the present invention. Further possible applications are opened up, for example, by generating beta-sheet proteins with new catalytic properties, for example resistance properties and fluorescent properties. An example of a protein whose fluorescent properties can be altered is GFP. The small proteins which are by nature highly stable are particularly suitable for the designing. Alteration of their surface generated, according to the invention and by way of example, new specific binding properties in the protein, with stability being retained.

A possible class of stable proteins, which was selected according to the invention and by way of example, is the crystallines. Crystallines which are the structural proteins of the eye lens are usually not subjected to cellular turnover and, consequently, have also extraordinary

stability properties (Mandal et al., 1987, Rudolph et al., 1990). Gamma-crystallines, a class of crystallines in vertebrates, are monomeric proteins with a molecular mass of approximately 22 kDa. The main structural motif of the gamma-crystallines is the antiparallel beta-sheet (Hazes and Hol, 1992, Richardson et al., 1992, Hemmingsen et al., 1994). Gamma-crystallines consist of two very similar globular domains, an N- and a C-terminal domain, which are linked to one another by a V-shaped linker peptide. The folding pattern characteristic for gamma-crystallines („greek-key" motif Slingsby, 1985, Wistow and Piatigorsky, 1988) is the most likely reason for the considerable thermostability and stability against denaturing agents (Mandal et al., 1987). Gamma-II-crystalline from calf eyes is a 21 kDa protein with, for its size, unusually many (7) cysteines which are in the reduced state under physiological conditions.

In its properly folded state, gamma-II-crystalline has no binding properties whatsoever. The inventive alteration (mutagenesis) of a selected solvent-exposed region of this protein, which consists of the beta-sheet structural motif, surprisingly resulted in alteration of the surface structure and charge pattern of the protein and thus in generation of new binding properties. In this connection, only regions or amino acid positions whose involvement in preserving the structure of the protein is insignificant were reacted. Mutagenesis of small beta-sheet proteins (Riddle et al., 1997) has shown that a high percentage of the protein is capable of forming the native beta-sheet structure correctly, despite considerable changes in the sequence.

Attempts at mutating particular protein regions with the aim of isolating molecules having improved or new binding properties exist already for recombinant antibody fragments (Nissim et al., 1994, de Kruif et al., 1995), for proteins with established binding properties (receptors, inhibitor proteins, DNA-binding proteins) and for peptide libraries (Cortese et al., 1995, Haaparanta and Huse 1995, McConell et al., 1996). In the case of antibodies, only the antigen-binding domains which are present as loop regions are mutagenized. This is likewise the case for most other proteins such as, for example, tendamistat (McConell and Hoess, 1995) or cytochrome b_{562} (Ku and Schultz, 1995). Here too, loop regions are mutagenized. Examples of mutageneses in alpha-helices are the Z-domain of protein A (Nord et al., 1997) and the zinc-finger domain CP-1 (Choo and Klug, 1995). The previous mutageneses merely altered the specificity of the binding and always started from proteins with already established binding properties. A protein without binding properties was never used, nor was

a beta-sheet structural motif specifically altered. In the method described here, for the first time a specific mutagenesis was carried out in the rigid beta-sheet region of a protein without any binding properties. This resulted, unexpectedly, in a protein with considerable stability and specific binding properties, comparable to antibody molecules.

A suitable system for isolating mutagenized beta-sheet proteins with *de novo* binding properties is the phage display system. This system makes possible very efficient screening of a large repertoire of protein variants for specific binding properties (Smith, 1985). In this connection, a protein variant is in each case presented on the surface of a filamentous phage and can interact with the target molecules immobilized on a solid phase. Proteins binding to the target molecule can be obtained by eluting the phages. After isolating the phage DNA, the DNA sequence of the specifically binding protein variants can be determined. In addition to the phage display system, it is also possible to apply other selection systems such as, for example, bacterial surface display (Stahl and Uhlen, 1997) or ribosome display (Hanes et al., 1997).

Using the above-described invention, it is surprisingly possible to alter, for example, the very stable beta-sheet protein gamma-II-crystalline by targeted, site-specific mutagenesis in the beta-sheet on the surface such that a protein with specific binding properties is generated from the non-binding protein. Randomizing eight amino acid positions thus leads, for the first time, to mutagenesis in a scaffolding molecule within a relatively rigid region of the protein. Thus a protein species which is „antibody-like" with respect to its specific binding properties is prepared from the beta-sheet protein gamma-II-crystalline. Gamma-II-crystalline or other small stable beta-sheet proteins can generally be used in the described method as novel scaffolding molecules for designing novel binding properties. The modelled beta-sheet proteins can replace, for example, recombinant antibodies in various applications. Due to their relatively small size (20 kDa), they are suited as fusion partners for other functional proteins (preparation of multifunctional proteins). Further possible uses are in gene therapy in which they can be employed as modules for cell-specific targeting of gene-therapy vectors and in intracellular immunization. Furthermore, beta-sheet mutants with catalytical properties can be used in enzyme application areas. The stability of the novel binding proteins makes additionally possible applications which cannot be carried out at present using recombinant antibodies, for example in human and veterinary medical diagnostics and therapy and in

biosensor and bios paration methods. Further fields of application are generally the pharmaceutical and cosmetic industries and the analysis and removal of harmful substances.

In the following, some preferred embodiments of the invention are described.

The proteins with beta-sheet structure, selected for mutagenesis according to the invention, have either no binding properties or no catalytic or enzymic activity or fluorescence properties or their activity, fluorescence properties or binding properties are such that an alteration, in particular improvement, is desirable.

Proteins with beta-sheet structure are known. An example of a protein class with beta-sheet is the crystallines, in particular alpha- beta- and gamma-crystallines. It is in principle possible to use crystallines from all kinds of animals, for example from vertebrates, rodents, birds and fish. Further examples of proteins which have beta-sheet structure and can be mutagenized according to the invention are: spherulins, heat shock proteins, cold shock proteins, beta-helix proteins, lipocalins, certins or transcription factors, fibronectins, GFP, NGF, tendamistat or lysozyme. For example, individual subunits or domains of the said proteins, for example crystallines, which have beta-sheet structure, are mutagenized according to the invention.

Among the crystallines, particular preferred mention must be made of gamma-crystalline for which it was possible, according to the invention and by way of example, to demonstrate that the beta-sheet structure thereof can be modified, i.e. mutagenized, such that new specific binding properties or new catalytic activities which are comparable to, for example, an antibody molecule are formed. An example of a gamma-crystalline is gamma-II-crystalline.

Examples of beta-helix proteins can be found, inter alia, in Jenkins J. et al., J. Struct. Biol. 1998, 122 (1-2): 236-46, Pickersgill, R. et al., J. Biol. Chem. 1998, 273 (38), 24600-4 and Raetz C.R. et al., Science 1995, 270 (5238), 997-1000.

The beta-sheet structure is defined by being essentially sheet-like and almost completely flat. In contrast to alpha-helices which are formed by a continuous part of the polypeptide chain, beta-sheets may be composed of various regions of the polypeptide chain. This makes it possible for regions which are relatively far apart in the primary structure to be located right next to one another. A beta-strand is typically 5-10 amino acids in length and is almost

completely flat. The beta-strands are so close to one another that hydrogen bonds form between the C=O group of one and the NH group of the other strand and vice versa. Beta-sheets may be composed of a plurality of strands and have a sheet-like structure. The C-alpha atom is located alternately above or below the sheet-like plane. The amino acid side chains follow this pattern and are thus orientated alternately upwards and downwards. Depending on the orientation of the beta-strands, a distinction is made between parallel and antiparallel sheets. According to the invention, both can be mutagenized and used for preparing the claimed proteins.

For mutagenesis of the beta-sheet structure, those beta-sheet regions in the protein, which are close to the surface, are selected. An amino acid exposed on the surface can be identified on the basis of the available X-ray crystal structure. If no crystal structure is available, it is possible by means of computer analysis to try to predict beta-sheet regions exposed on the surface and accessibility of individual amino acid positions on the basis of the available primary structure (www.embl-heidelberg.de/predictprotein/predictprotein.html) or to model the 3D protein structure (www.expasy.ch/swissmo/SWISS-MODEL.html) and thus to obtain information about amino acids possibly exposed on the surface.

However, beta-sheet mutageneses for which a time-consuming preselection of the amino acid positions to be mutagenized can be dispensed with are also possible. Those DNA regions which code for the beta-sheet structures are isolated from their DNA environment, subjected to a random mutagenesis and subsequently re-integrated into the DNA coding for the protein, from which they have been removed previously. This is followed by a selection method for mutants having the desired binding properties and/or catalytic properties and/or fluorescence properties.

In another embodiment of the invention, the beta-sheet regions close to the surface are, as already described above, selected and the amino acid positions to be mutagenized are identified within these selected regions. These amino acid positions selected in this way can then be mutagenized at the DNA level either targeted, i.e. a codon coding for a particular amino acid is replaced by a codon coding for a different, previously selected specific amino acid, or this exchange is carried out within the framework of a random mutagenesis, with the amino acid position to be exchanged being defined but not the codon coding for the new, hitherto undefined amino acid.

Amino acids exposed on the surface are accessible to the surrounding solvent. If the accessibility of amino acids in a protein is more than 8% compared with the accessibility of the amino acid in the model tripeptide Gly-X-Gly, these amino acids are called amino acids exposed on the surface. These protein regions or individual amino acid positions are also preferred binding sites for possible binding partners which are to be selected for according to the invention. The binding partners may be, for example, antigens or substrates or substrate-transition-state analogues.

According to the invention, it is possible to mutagenize virtually all proteins which display beta-sheet structures located on the surface and accessible to a solvent or a binding partner. To this end, suitable proteins are mainly those which are particularly stable, i.e. resistant to denaturation, for example, or sufficiently „small".

„Mutagenization" means according to the invention the alteration of one or more amino acids exposed on the surface in the polypeptide chain with beta-sheet structure. This includes, for example, amino acid substitutions in which an amino acid with particular properties with respect to its polarity, charge, solubility, hydrophobicity or hydrophilicity is replaced by an amino acid with a different property, thus for example a non-polar, hydrophobic amino acid by a polar amino acid, a negatively charged amino acid by a positively charged amino acid, etc. The term „mutagenization" also comprises insertions and deletions of one more amino acids. A precondition is that the mutations comprise amino acids exposed on the surface in at least two beta-strands exposed on the surface of at least one beta-sheet exposed on the surface. The mutations are preferably and specifically introduced at individual amino acid positions in the beta-sheet or in selected regions of the beta-sheet. Mutagenizations may be present in one region or in a plurality of regions of the beta-sheet structure. The alterations may comprise adjacent amino acids or amino acids which are relatively far apart in the beta-sheet. The alterations may also comprise amino acids in various beta-sheets, i.e. in more than one beta-sheet. The insertions, deletions or substitutions of one or more amino acids are located in at least two beta strands exposed on the surface of at least one beta-sheet exposed on the surface. In this connection, it is possible to substitute, delete or insert one or more amino acids in one beta strand exposed on the surface, i.e. one beta strand exposed on the surface can have a plurality of mutations, if at least two beta strands exposed on the surface are mutated. In a further embodiment, in each case one beta-strand exposed on the

surface of at least two beta-sheets exposed on the surface is mutagenized, i. . one beta-sheet exposed on the surface has in each case at least one mutagenized beta-strand exposed on the surface. In another embodiment of the invention, the mutagenized beta-sheets exposed on the surface are arranged antiparallel to one another, and preference is given to at least two antiparallel arranged beta-sheets.

According to the invention, it is preferred, for example, that two or there beta-strands exposed on the surface are mutagenized. According to the invention, it is also possible that four or more beta-strands exposed on the surface are mutagenized. Furthermore, it is possible that at least two beta-strands in at least two beta-sheets are mutagenized, with preference being given to mutagenesis of three beta-strands in two antiparallel beta-sheets.

In one embodiment of the invention, mutagenesis is carried out by assembling DNA oligonucleotides having the amino acid codon NNK. It is, of course, also possible to use other codons (triplets).

The mutations are carried out such that the beta-sheet structure is retained. In general, mutagenesis takes place on the outside of a stable beta-sheet region exposed on the surface of the protein. It comprises both site-specific and random mutagenizations. Site-specific mutageneses which comprise a relatively small region in the primary structure (approx. 3-5 amino acids) can be carried out using the commercially available kits from Stratagene (QuickChange) or Bio-Rad (Muta-Gene phagemid in vitro mutagenesis kit) (cf. US-A-5,789,166; US-A-4,873,192).

If larger regions undergo site-specific mutagenesis, a DNA cassette has to be prepared, and the region to be mutagenized is obtained by assembling oligonucleotides containing the mutated and the unaltered positions (Nord et al., 1997; McConell and Hoess, 1995). Random mutageneses can be introduced by propagating the DNA in mutator strains or by PCR amplification (error-prone-PCR) (e.g. Pannekoek et al., 1993). In this case, a polymerase with increased error rate is used. In order to increase the extent of the introduced mutagenesis or to combine different mutations, it is possible to combine the mutations in the PCR fragments by means of DNA shuffling (Stemmer, 1994). The review by Kuchner and Arnold (1997) provides an overview of these mutagenesis strategies for enzymes. In order to

carry out the said random mutagenesis in a selected DNA region, a DNA cassette which is utilized for the mutagenesis has to be constructed here, too.

The DNA molecules obtained in the mutagenesis step are expressed in a suitable expression system. Preference is given to those expression systems which facilitate subsequent selection and isolation of mutants having the desired binding properties and/or the desired catalytic or enzymic activity. Such expression vectors and expression systems are known to the skilled worker and have been described already in more detail above. Of course, it is also possible to use other expression systems which allow inventive selection for mutants with specific properties or activities.

Preference is given to using for expression and selection the phage display system in which all mutants produced at the DNA level are cloned into a phagemid and expressed on phage surfaces. In the case of proteins containing reduced cysteines, it is possible, in a particularly preferred embodiment of the invention, to add GSH, in order to improve exposition and selection of the mutants.

The invention includes the mutagenized proteins, DNA molecules, RNA molecules derived thereof and functional parts thereof which code for a protein which has a mutagenized beta-sheet structure and is capable of binding to a desired binding partner in a new or altered manner or which can have a new or altered catalytic activity for a substrate or new or altered fluorescence properties. The term „functional parts" relates to subunits, domains and epitopes of the protein with beta-sheet structure, which have been mutagenized according to the invention and possess the desired binding properties and activities or are partly responsible therefor.

Mutants having the desired binding properties and/or the desired catalytic activities and/or fluorescence properties are selected and isolated in a manner known per se. Examples of selection methods and isolation methods for mutants having new or altered binding properties and new or altered catalytic activities are described below:

When selecting for desired binding properties, the mutated proteins or functional parts thereof are contacted with their binding partners. Suitable detection methods select mutants having the desired binding properties.

When selecting for catalytic activity, the mutated proteins or functional parts thereof are connected with the substrates and then selected for the desired enzymic activity by suitable detection methods.

Catalytic activity can be selected for in several ways:

1. Phage display:
Coupling of transition-state analogues to a solid phase and selecting the mutant library for the said analogues. These substances are analogues to transitional states of the substrate, which typically form during enzymic conversion of a substrate to the product (substrate-transition-state product). For this, however, the transition state of the substrate must be known. It is also possible to carry out a screening for substrate binding.

2. Without phage display:
Cloning of the mutants into a bacterial expression system and plating of the recombinant bacteria for forming individual colonies. The mutated protein can be expressed in the bacteria by adding inducers (e.g. IPTG) to the nutrient medium. The nutrient medium must furthermore contain the substrate whose conversion is to be screened for. The substrate must form an identifiable, e.g. coloured, product during conversion. Those bacteria which express a mutant converting the substrate in the nutrient medium require a different colour. An example would be the screening for beta-galactosidase activity and conversion of X-Gal (blue staining) (Zhang et al., 1997).

3. The skilled worker knows further detection methods:
Apart from the colour formation variant, it would also be possible, to select, for example, protein mutants which mediate a new resistance (addition of antibiotics to the nutrient medium) or which make possible growth on minimal nutrient media on which the „normal" bacterium will not grow. It is possible here to make use of the selective growth advantage of the bacteria having the new protein mutant (Crameri et al., 1997).

4. Expression and secretion of the mutated proteins:

For example in bacteria, obtaining the supernatant and testing for the desired enzymic activity to be selected (You and Arnold, 1996). The present invention thus solves the problem of generating proteins having new binding properties or new catalytic properties by mutagenizing proteins with beta-sheet structures in this structural motif. Those proteins are selected for, which possess the desired new or altered, preferably improved binding properties or the desired new or altered, preferably improved, enzymic or catalytic activities. The system of the invention even makes it possible to alter beta-sheet proteins which have no binding properties or no enzymic properties such that, after mutagenization in the beta-sheet, they acquire binding properties or catalytic properties.

According to the invention, „binding properties" means, for example, the specific affinity of an antigen for an antibody. After the mutagenesis has been carried out according to the invention, the beta-sheet protein thus possesses antibody-like properties and combines the advantages of the high binding specificity of an antibody with the advantageous stability properties of a beta-sheet protein. The beta-sheet proteins with antibody-like properties, prepared according to the invention, may also possess a catalytic function.

However, the solution proposal according to the invention makes it also possible to generate proteins with beta-sheet structure, which have new or altered catalytic activities. The alteration of other protein properties, for example the fluorescent properties of GFP, would also be possible.

According to the invention, alteration of the binding properties, the catalytic activity or the fluorescence properties means both a deterioration and an improvement in the said properties, with an improvement being preferred.

According to the invention, a „protein having a new specific property" or „having a new catalytic activity" means a protein which previously has not had any specific binding property or catalytic activity and now has a specific binding property or a catalytic activity or a combination of both, due to the specific mutagenization of amino acids exposed on the surface in at least two beta-strands exposed on the surface of at least one beta-sheet exposed on the surface. However, this also includes proteins which already had a specific binding property or a catalytic activity prior to mutagenization and, after mutagenization in the beta-sheet, possess another, additional specific binding property and/or catalytic activity. It

is, of course, also possible that a protein with a specific binding property now has a catalytic activity or vice versa.

The invention furthermore comprises those proteins which already possess a specific binding property and/or an enzymic or catalytic activity and/or fluorescence properties and which, after mutagenization of amino acids exposed on the surface in at least two beta-strands exposed on the surface of one or more beta-sheets exposed on the surface, obtain an improvement in, or, in more general terms, and alteration of their specific binding properties and/or their catalytic activity and/or their fluorescence properties.

In this respect, the method of the invention and the proteins prepared thereby also differ from proteins and methods from the prior art, in which the beta-sheet structure was altered by random mutagenizations which were not directed towards the beta-sheet structure but towards the entire protein and which were in particular not targeted towards amino acids exposed on the surface in at least two beta-strands exposed on the surface of at least one beta-sheet exposed on the surface or which related to such amino acids exposed on the surface.

In a preferred embodiment of the invention, which will be described by way of example below, gamma-crystalline, as in the example of a protein with beta-sheet structure, was chosen as starting point for the mutagenesis. To this end, first amino acid positions exposed on the surface were selected through structural studies and mutagenized by mutagenization methods known per se. The mutants obtained were expressed in a suitable, likewise known expression system. The selection was directed towards those mutants whose amino acids exposed on the surface in the beta-sheet of the gamma-crystalline showed specific binding towards the antigen BSA-estradiol 17-hemisuccinate. Although a plurality of mutants having the desired binding property were isolated, only one carries the expected amino acid exchanges. Thus, an antibody-like non-immunoglobulin molecule was obtained, which is based on the starting protein gamma-crystalline.

The method of the invention makes it possible to prepare an indeed enormous number of mutants. Mutagenesis of just eight amino acid positions makes it possible to form 2.6×10^{10} different protein species which can be analysed for the desired binding properties and catalytic activities.

According to the invention, it was furthermore shown that the fluorescence properties of a protein with beta-sheet structure can be altered by mutagenesis of amino acids exposed on the surface.

The mutated genes obtained can be propagated in suitable systems and the proteins can be expressed. Suitable expression systems are prokaryotic or eukaryotic systems. The DNA coding for the mutated protein is transferred, for example, into a suitable vector, for example into an expression vector, and introduced into a host cell by transformation, transfection or infection. The linkage to regulatory sequences which specifically control expression of the heterologous mutated DNA is advantageous, of course.

A host cell which may be used is a host cell of a higher eukaryote, for example a mammalian cell, or of a lower eukaryote, for example a yeast cell, or a prokaryotic cell, for example a bacterial cell. An example of a possible bacterial host cell is E.coli or B.subtilis. Cell-free translation systems for preparing the proteins by using RNA which is derived from the DNA of the present invention are also possible. Suitable cloning and expression systems are described in various textbooks for molecular biology, biotechnology and gene technology. Examples include Sambrook et al., 1989 and Ausubel et al., 1994.

The invention, described in general terms above, is illustrated in more detail below on the basis of an exemplary embodiment and the attached drawings. The example is to be understood as a possible form of the invention and the invention is not restricted to this particular embodiment.

The attached figures show:

Fig. 1: Oligonucleotides for assembling the gamma-crystalline mutants.

Fig. 2: Schematic representation of oligonucleotide assembling and subsequent PCR on streptavidin-loaded magnetic beads (MB). The positions marked with X indicate the randomized amino acid positions.

Fig. 3: Schematic representation of the amplification of the non-mutagenized region of gamma-II-crystalline.

Fig. 4: Oligonucleotides for amplifying the non-mutagenized region of gamma-II-crystalline.

Fig. 5: Schematic representation of the pCANTAB 5E- gamma-II-crystalline expression cassette. g3-SS: signal peptide sequence of the phage protein G3; E-tag: 11 amino acids for immunological detection; fd Gen 3: minor coat protein 3 of the filamentous phage M13.

Fig. 6: Polyclonal phage ELISA with concentrated phages after 3rd panning. The microtitre plates were coated either with the BSA-beta-estradiol 17-hemisuccinate conjugate or just with BSA as control. Shown next to one another are the binding of gamma-II-crystalline wild-type phages (GC-WT), of the phages from the starting library (GCUC-1) and of the phages concentrated by repeated panning (E-17 phages) to the particular antigen.

Fig. 7: Partial DNA sequence of the BSA-estradiol-17-hemisuccinate-binding gamma-II-crystalline mutant 12A (Mu 12A) in phagemid pGCKT 8-3 and of gamma-II-crystalline wild-type (WT) in pCANTAB 5E respectively. The introduced cleavage sites Sfi I (5') and Bst EII (3') are indicated by italics and underlining. The codons of the randomized amino acid positions are in bold type.

Fig. 8: Derived amino acid sequences of the BSA-estradiol-17-hemisuccinate-binding gamma-II-crystalline mutant 12A (Mu 12A) and of gamma-II-crystalline wild-type (WT) after expression in the phagemids and removal of the signal peptide. The randomized amino acid positions are indicated by bold type and amino acids which have actually been exchanged are indicated by bold type and are underlined. Amino acids additionally introduced at the N-terminals via the Sfi I cleavage site and the C-terminal E-tag fusion are shown in italics and underlined.

Fig. 9: Sequence of the primers used for cloning Mu 12A and gamma-II-crystalline into vector pET-20b.

Fig. 10: Derived protein sequence of the BSA-estradiol-17-binding mutant 12A and of gamma-II-crystalline after expression in pET-20b. The randomized amino acid positions are indicated by bold type and amino acids which have actually been exchanged are indicated by bold type and are underlined. C-terminal amino acids additionally introduced via the cloning, including the 6 histidine are shown in italics and underlined.

Fig. 11: Concentration-dependent binding of mutant 12A to the BSA-beta-estradiol-17-hemisuccinate conjugate. The binding of the mutant (12A) and of gamma-II-crystalline (WT) to the conjugate (BSA-Estr. 17) and, as a control, to BSA was analysed.

Fig. 12: Stability of mutant 12A against the denaturing agent guanidine. The figure shows the emission maxima after incubating the purified mutant 12A and gamma-II-crystalline proteins with various concentrations of guanidine for various periods.

Fig. 13: Fluorescence emission spectrum of wild-type gamma-crystalline and mutant 12A in 50 mM Na phosphate, pH 6.5. The fluorescence signal (Fig. 13A) was measured at an excitation wavelength of 280 nm. The protein concentration was 100 µg/ml. Fig. 13B shows the absorbence spectra of the protein samples used for fluorescence measurement. The absorbence was determined in a cuvette with 1 cm path length.

EXAMPLE

Preparation of a gamma-crystalline mutant with specific binding to the hormone estradiol

The design of novel beta-sheet proteins with antigen-binding properties is shown on the basis of isolating a mutant of the bovine gamma-B-crystalline (gamma-II), which binds specifically to the hormone estradiol. Specific alteration of selected amino acid positions of a

beta-sheet exposed on the surface produced a novel stable prot in with beta-sheet structure and specific binding properties. After selecting the beta-sheet region or amino acids suitable for mutagenesis, a site-specific mutagenesis was carried out at the DNA level, and in a phagemid a beta-sheet mutant library was prepared, which makes expression and subsequent selection for novel binding properties of the mutants in the phage display system possible. The isolated mutant was compared to the starting protein gamma-II-crystalline with respect to its new properties.

Selection of a suitable region for mutagenesis in gamma-crystalline

Based on the X-ray structure of gamma-II-crystalline (Wistow et al., 1983), the N-terminal domain of gamma-II-crystalline (Acc. M16894) was selected for mutagenesis. Eight amino acids in all, which form a continuous surface segment, were identified there. The selected amino acids are part of a beta-sheet and should not contribute substantially to preserving the structure. They are amino acid positions which are accessible to the solvent and thus also to possible binding partners. The eight amino acids Lys 2, Thr 4, Tyr 6, Cys 15, Glu 17, Ser 19, Arg 36 and Asp 38 comprise an area of approx. 6.1% of the total surface area of the protein.

Preparation of a DNA pool of mutated gamma-II-crystalline genes

The eight amino acid positions were randomized by site-specific mutagenesis. This makes it possible to produce 2.6×10^{10} different protein species. The region to be mutagenized was obtained at the DNA level by assembling individual oligonucleotides. This was followed by cloning into a phagemid constructed for selection in the phage display system.

Oligoassembling

For mutagenesis, 227 bp containing the 5' region of the gamma-crystalline mutants with the eight randomized amino acid positions and also suitable restriction cleavage sites were assembled on a solid phase. 10 individual oligonucleotides in all were used therefor, three of which contained the randomized amino acid positions (Fig. 1). During primer synthesis, the nucleotide mixture NN(T/G) was used at the eight positions to be mutagenized, resulting theoretically in 32 different codons at one position (cf. Nord et al., 1997). At the start of the

assembling, biotinylated oligonucleotides were attached to streptavidin-loaded magnetic beads (MBs) from Dynal (M-280). After several attachment, ligation and polymeration steps, it was possible to amplify the pool of mutagenized regions of gamma-crystalline, assembled on the solid phase, by PCR (Fig. 2). The PCR products of approx. 250 bp in length contained an Sfi I cleavage site 5' and a Bst EII cleavage site 3'.

All oligonucleotides used for assembling were adjusted to a concentration of 100 pmol/µl. First, the primers GCLIE1B and GCLIE2P were assembled. For this, 36 µl of washing and binding buffer (WB buffer: 1M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) were added to in each case 4 µl of the primers and the mixture was incubated at 70°C for 5 min. After assembly of the two primers and further incubation at 70°C for 5 minutes, the primer mixture was slowly cooled to room temperature. 4 µl of the GCLIE1B/GCLIE2P primer hybrids were mixed with 56 µl of WB buffer and added to 300 µg of the streptavidin-loaded MBs which had been washed beforehand with washing and binding buffer. Incubation at room temperature for 15 minutes was followed by washing the MBs with WB buffer and TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). A primer linker fragment is added to the MBs coupled to the first primer hybrid, which fragment is prepared as follows: 4 µl of primer GCLIB4P or GCLI5P are mixed with 36 µl of 1 x ligation buffer from GIBCO BRL (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000). After incubation at 70°C for 5 minutes, both mixtures are combined, incubated at 70°C for a further 5 min and cooled to room temperature. After adding 12 units of T4 DNA ligase (GIBCO BRL) and 8 µl of 1 x ligation buffer, the reaction mixture is incubated at room temperature for 1 h. 12 µl of this GCLIE3P/GCLIB4P/GCLI5P bridging fragment are admixed with 54 µl of 1 x ligation buffer and 6 units of ligase, and the mixture is added to the washed MBs containing the first primer hybrid and incubated at room temperature for 1 h. After the ligation reaction, the MBs are washed twice with TE buffer and taken up in 64 µl of1 x ligation buffer containing 8 µl of ligase. 8 µl of the assembled primer mixture GCLI6P/GCLIB7P, which primers have been assembled beforehand in analogy to those of GCLIB4P/GCLI5P, were then added to the MBs. The ligation was again carried out at room temperature for 1 h. After washing the MBs twice in TE buffer, 12 µl of the 2nd bridging fragment GCLIB8P/GCLIE9P/GCLIE10 are added and the mixture is ligated for 1 h. The 2nd bridging fragment is prepared analogously to the first bridging fragment, GCLIE9P and GCLIE10 being assembled first and then ligated with GCLI8P in the second step. The MBs with the immobilized primers are then again washed with TE buffer. The subsequent DNA-polymerase and ligase reaction fills in the gaps in the second strand. The MBs are incubated at 37°C for 30 min in the following buffer mixture:

52.5 µl of H_2O, 6 µl of buffer L from Boehringer (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM dithioerythritol), 0.5 µl of dNTPs (25 mM of each dNTP) and 1 µl (2 units) of Klenow fragment (Boehringer). Washing the MBs twice with TE buffer is followed by the ligation reaction at room temperature for 1 h. A 100 µl mixture contains 10 units of ligase. After two washing steps with TE buffer, the DNA strand non-covalently bound to the MBs is removed by treatment with 40 µl of 0.1 M NaOH for 30 s, and the MBs are resuspended in 60 µl of TE. The PCR for amplifying the library is carried out using the MBs as template. The PCR reaction mixture (50 µl) is prepared as follows: 6 µl of MBs, 5 µl of 10 x PCR reaction buffer from Stratagene (100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl pH 8.75, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/ml BSA), 1 µl (2,5 units) of Pfu DNA polymerase (Stratagene), 0.5 µl of dNTPs (25 mM of each dNTP), 0.35 µl of GCLIE1B. 0.35 µl of GCLIA11B and 36.8 µl of H_2O. The PCR was carried out in 35 cycles with primer annealing at 55° for 1 min, a polymerase reaction at 72°C for 1.5 min, denaturation at 95°C for 1 min and a final polymerase reaction at 72°C for 5 min.

Preparation of phagemid pGCKT 8-3

Starting from phagemid pCANTAB 5E (PRAS kit from Pharmacia Biotech), a phagemid derivative for cloning the gamma-II-crystalline mutant band was thus constructed. The entire 3' region of gamma-II-crystalline (C-terminal domain) and the non-mutagenized 5' region were amplified by means of PCR using plasmid pGII (Mayr et al., 1994) as template and primers GCFORNOT and GCBACKSfiBst (Figs. 3, 4).

The Sfi I (GCBACKSfiBst) and Not I (GCFORNOT) cleavage sites introduced by the primers make insertion of the PCR product into phagemid (GCFORNOT) pCANTAB 5E possible. Together with the GCBACKSfiBst primer, a Bst EII cleavage site was additionally integrated into the gamma-crystalline gene, which allowed cloning of the mutated gamma-crystalline DNA fragments. *de novo* introduction of the cleavage site does not alter the amino acid sequence in gamma-II-crystalline. After sequencing, the PCR product was cloned as Sfi I/Not I fragment into phagemid Sfi I/Not I cut with pCANTAB 5E. The phagemid pGCKT8-3 constructed in this way was the starting point for preparing the gamma-II-crystalline phage display library.

Preparation of the gamma-crystalline mutant-library and cloning of wild-type gamma-II-crystalline

Phagemid pGCKT 8-3 was cut with Bst EII and Sfi I restriction enzymes and subjected to phosphatase treatment (shrimps phosphatase from USB). After the individual cleavages, the DNA was fractionated by gel electrophoresis, and the cleaved vector fractions were excised and isolated from the agarose gels by means of electroelution. Any further enzymatic treatment was preceded by phenol/chloroform extraction and precipitation of the DNA with glycogen. The DNA fragment pool which had been amplified by means of PCR and which contained the mutated region of gamma-II-crystalline was cleaved with Sfi I and Bst EII restriction enzymes. A total 440 ng of phagemid and 110 mg of PCR product were used for ligating the PCR products into the prepared pGCKT 8-3 phagemid. The ligations were carried out with a total 44 units of T4 DNA ligase (GIBCO BRL) in 20 µl mixtures at 16°C overnight. After inactivating the ligase at 70°C for 20 minutes, the ligation reactions were desalted by drop dialysis for 1 h. In each case 30 µl of electrocompetent *E. coli* TG 1 cells were transformed with in each case 15 µl of the dialysed ligations. The electrocompetent cells were prepared and transformed as described in the PRAS-kit manual. The transformed cells were created onto glucose- and ampicillin-(100 µg/ml) containing SOBAG plates (see PRAS-kit manual from Pharmacia-Biotech) and incubated at 30°C overnight. The GCUC-1 library prepared contained 250 000 original clones. The clones were washed off with 2 x YT medium (see PRAS-kit manual) containing 1% glucose and 20% glycerol, aliquoted and stored at -80°C. The amplification factor of the library was determined to $7x10^6$. The proportion of recombinant clones in the GCUC-1 library was 97%. Sequencing of randomly selected clones revealed that codons were used wit the expected variants at the randomized amino acid positions. Expression rates of 30-60% were detected in the library by means of Western-blot analyses.

In control experiments, gamma-II-crystalline DNA was amplified using primers GCFORNOT (5' GAGTCATTCTGCGGCCGCATAAAAATCCATCACCCGTCTTAAAGAACC 3') and GCBACKSFI (5' CATGCCATGACTCGCGGCCCAGCCGGCCATGGGGAAGAT CACTTTTTACGAGGAC 3') and plasmid pGII (Mayr et al., 1994) as template. After cleavage with Not I and Sfi I restriction endonucleases, the sequenced PCR product was cloned into the Sfi I/Not I phagemid likewise cut with pCANTAB 5E .

A phage display design and selection for novel binding properties

The commercially available phage display system PRAS from Pharmacia-Biotech was used for selecting gamma-crystalline mutants for binding properties. In the pCANTAB 5E (wild-type gamma-II-crystalline) and pGCKT 8-3 (gamma-crystalline mutants) phagemids used, the gamma-crystallines are fused N-terminally to the G3 signal peptide and C-terminally to an E-tag which makes immunological detection of the proteins possible (Fig. 5). Depending on the bacterial strain used, the *amber* stop codon after the E-tag is either recognized (*E. coli* HB 2151), and cleavage of the signal peptide is followed by secretion or overreading of the *E. coli* cell. After adding a helper phage, recombinant phages can be formed which expose the gamma-II-crystalline variants on their surface.

Optimization of cultivation conditions for the GCUC-1 library and gamma-II-crystalline wild-type phages

Under the cultivation conditions described in the PRAS manual, it was not possible to detect in any of the recombinant phages the expected fusion proteins (gamma-II-crystalline/protein 3) by means of Westerb-blot analyses. Only the addition of reduced glutathione (GSG) during phage formation altered the redox state in the periplasm of the bacterial cell and thus prodived more favourable conditions for phage assembling. When using the gamma-II-crystalline clone, it was possible to detect recombinant phages carrying the fusion protein only with the addition of GSH. Increasing GSH concentration also increased the proportion of gamma-II-crystalline phages. The optimal GSH concentration was determined to 8 mM. One reason for poor gamma-crystalline expression on the phage surface in the absence of GSH could be the high proportion of reduced cysteines (7) in gamma-crystalline. When the partically unfolded gamma-crystalline enters the periplasm, it could, under the oxidative conditions prevailing there, misfold and form aggregates due to the formation of disulphide bridges. This could also suppress phage assembling. When using proteins with reduced cysteines in the phage display system, it may be possible to improve formation of recombinant phages generally by adding GSH.

Selection process using the GCUC1 phage display library

To screen the GCUC-1 library, all glass equipment used was sterilized at 220°C for 4 h and plastic material was sterilized with Helipur for 1 h. GCUC-1 library panning was carried out using BSA-beta-estradiol 17-hemisuccinate (Sigma) as antigen and microtitre plates (Maxisorp from NUNC) as solid phases. During the 3 rounds of panning, the stringency of the washing steps was increased. For the first cultivation, 100 ml of 2 x YT medium containing 2% of glucose and ampicillin (100 µg/ml) were inoculated with 50 µl of the GCUC-1 library. The bacteria grew at 37°C and 300 rpm to an OD_{600} of 0.4. 800µl of M13KO7 helper phage (1×10^{11} pfu/ml, GIBCO BRL) were added to 10 ml of this bacterial culture. This was followed by incubation at 37°C for 30 min without and for a further 30 min with gentle agitation (50 rpm). The bacterial pellet was obtained by centrifugation at room temperature and 1 500 rpm (Sorvall SS 34 Rotor) for 20 min and taken up in 100 ml of 2× YT medium containing 8 mM GSH, 100 µg/ml ampicillin and 50 µg/ml Kanamycin. The recombinant phages were produced by overnight culturing at 30°C and 300 rpm. The supernatant containing the recombinant phages was obtained by two centrifugations at 10 800g for in each case 15 minutes and subsequent filtration (pore size 0.45µm). The phages were concentrated by adding 1/5 of PEG/NaCl solution (20% PEG-8000, 2.5 M NaCl) to the supernatant, incubating on ice for one hour and two centrifugations at 4°C and 3 300g for in each case 30 minutes. The phage pellet obtained was suspended in 4 ml of PBS pH 7.2, and remaining cell components were removed by centrifugation (10 min, 11 600 g, room temperature). For the selection process (panning), 1 ml of concentrated phages were mixed with 1 ml of a 6% strength BSA solution (6% BSA in PBS, pH 7.2) and incubated at room temperature for 10 min. In each case 100µl of the phages treated in this way were added to the antigen-coated microtitre plate wells prepared as follows. NUNC-Maxisorp microtitre plates were coated with the antigen BSA-beta-estradiol 17-hemisuccinate. In each ases 100 µl of antigen solution (100 µg/ml in PBS pH 7.6) were introduced into 10 wells in total. The wells coated at room temperature overnight were washed three times with PBS, pH 7.6. Free binding sites were saturated by filling the wells with a 3% strength BSA/PBS solution, pH 7.2, at room temperature for 2 h. Prior to adding the BSA-treated phages, the wells were washed twice with a PBS solution (ph 7.2). Panning was carried out by agitating the microtitre plate gently (20 rpm) for 30 minutes followed by incubation without shaking at room temperature for 90 minutes. Unspecifically bound phages were removed by washing 10 times with PBS, pH 7.2/0.1% Tween-20 and washing 10 times with PBS, pH 7.2. Bound

phages were eluted by adding in each case 100 µl of 100 mM triethylamine (freshly prepared) per well and incubating at room temperature for 10 minutes. The base-eluted phages (1 ml) were neutralized by adding 500 µl of 1 M Tris-HCl pH 7.4. 750 µl of these phages were used for infecting 9 ml of TG-1 cells cultivated on minimal medium plates and having an OD_{600} of 0.4-0.5. For this, the bacteria were incubated with the phages at 37°C for 30 min. It was possible to save phages which had bound particularly tightly and had not been removed from the microtitre plate by triethylamine treatment by direct infection of TG-1 cells. For this, in each case 100 µl of the cultivated TG-1 cells were added to the wells. After incubating at 37°C for 30 minutes, the infected TG-1 cells were removed and combined with those from infection with the eluted phages. The infected bacteria were created onto 16x16 cm SOBAG plates and incubated at 30°C overnight. In each case 1µl of concentrated and eluted phages was used for determining the titre. The bacterial clones obtained were washed off the SOBAG plates with 12.5 ml of 2 x YT, 20% glycerol. The second and third pannings were carried out similarly to the first with the following changes. Phage cultivation was repeated using 20 µl of the washed-off library in 20 ml of medium. 2 ml of the cultivated bacterial culture were used for infection with the helper phage (bacterial/phages weight ratio: 1/20). In the seecond panning the microtitre plates were washed first 15 times with PBS/Tween-20 and then 10 times with PBS and in the 3rd panning first 20 times with PBS/Tween-20 and then 10 times with PBS.

ELISA for checking concentration and specific binding

Concentration of the phages specifically binding to the antigen was detected using a polyclonal phage ELISA. In addition to the eluted phages, phages of the staring library GCUC-1 and of wild-type gamma-II-crystalline were assayed for comparison. NUNC-Maxisorp plates were coated with 100 µl of BSA-estradiol17-hemisuccinate or BSA at a concentration of 2 µg/ml of PBS pH 7.6 at room temperature overnight. 3 washings of the wells with PBS, ph 7.6 were followed by blocking with 3% dried milk powder (Glücksklee)/PBS, pH 7.2 at 37°C for 2 h and another (3) washings with PBS, pH 7.6. The non-concentrated recombinant phages isolated after phage cultivation were firstly blocked at room temperature for 1 h (1:1 mixture with 6% strength dried milk powder (Marvel)/PBS pH 7.6. 100 µl of the blocked phages were applied per well and incubated at 37°C for 1 h.

Washing the wells in each case 3 times with PBS/Tween-20 and PBS was followed by incubation with the anti-M13 antibody-POD conjugate (Pharmacia-Biotech, dilution 1:5 000 in 3% Glücksklee/PBS) at 37°C for 1 h. After washing the plates, the enzyme-bound antibody was detected using 100 µl of Immuno-Pure-TMB substrate (Pierce). The colour reaction was stopped by adding 100 µl of 2M H_2SO_4 and extinction at 450 nm was determined. The result of the concentration of the phages binding to the BSA-estradiol conjugate, after the 3rd panning, is shown in Fig. 6.

Isolation and characterization of individual phages with specific binding to the conjugate

80 individual clones were selected from the bacterial clones obtained after the 3rd panning. Phages were isolated from the clones and assayed individually in the monoclonal phage ELISA with respect to their antigen binding. Individual bacterial clones were cultivated in 100 µl of 2 x YT medium containing 2% glucose and 100 µg/ml ampicillin in polypropylene microtitre plates (NUNC) with gentle agitation (100 rpm) overnight. 2 µl of these bacterial cultures were diluted 1:100 in the same medium and cultured at 100 rpm at 37°C to an OD_{600} of 0.4. Phages were obtained as described for the selection process. Deep well polypropylene microtitre plates from TECAN were used for phage cultivation. For the ELISA, 200 µl of the phage supernatant obtained after centrifugation (not concentrated) were blocked with 40 µl of 6xPBS/18% at room temperature for 1 h. 30 out of 80 clones assayed showed significant binding of the recombinant phages to BSA -Estradiol-17 and not to BSA assayed in parallel. Phages with wild-type gamma-II-crystalline showed in a control experiment no binding to BSA-estradiol-17 whatsoever. 14 selected binding phages were sequenced using the IRD 800-labelled primers pCANR1LAB (5' CCATGATTACGCC-AAGCTTTGGAGCC 3') and GCLISEQ (5' CTGAAAGTGCCGGTGTGTTGC 3'). Only in one case, sequencing revealed a gamma-crystalline variant (Mu 12A) which was mutated exclusively in the eight randomized amino acid positions. A number of clones showed shifts in the reading frame and, although theoretically coding for a functional protein, had alterations which were not exclusively in the expected gamma-crystalline region. These frame shift mutants were not studied further.

Characterizati n of beta-sheet mutant 12A

Expression of the fusion protein Mu 12A-minor coat protein 3 on the surface of the recombinant phages and expression of Mu 12A in *E. coli* strain HB 2151 were detected by means of Western-blot analyses using the anti-G3P and anti-E-Tag antibodies (Pharmacia-Biotech), respectively. The DNA sequences of mutant 12A in phageimid pGCKT 8-3 and of gamma-II-crystalline wild-type are depicted in Fig. 7. The DNA sequence starts at the Sfi I cleavage site which is already present in the starting phageimid pCANTAB 5E and ends, in the case of pGCKT 8-3, at the Bst EII site newly introduced into the gamma-II-crystalline gene and, in the case of the gamma-II-crystalline wild-type gene, at the original sequence. Fig. 8 depicts the amino acid sequences derived therefrom. Codon randomization at amino acid position 36 does not change the amino acid arginine at this position. Computer modelling of mutant 12A shows that the amino acid exchanges do not cause large alterations in the protein structure compared with the starting protein. However, the net charge becomes more positive.

Expression of Mu 12A in pET-20b

In order to characterize mutant 12A in detail, the DNA was recloned into plasmid pET-20b (Novagen). The plasmid makes possible a high expression of the recombinant DNA in *E. coli* strain BL 21 and simple purification of the foreign proteins. Genes are expressed here without signal peptide and with a C-terminal fusion of 6 histidine residues. The DNAs of mutant 12A and of gamma-II-crystalline wild-type were amplified by means of PCR using the appropriate phageimid DNA and primers GC 20bback12A/GC for 20b for mutant 12A and GC 20bbackWT/GC for 20b for the wild-type (Fig. 9). The PCR fragments were cleaved with restriction endonucleases Nde I and Bam HI and cloned into vector pET 20b cut with Nde I/ Bam HI. Fig. 10 depicts the theoretical amino acid sequence of mutant 12 A and of gamma-II-crystalline, respectively, after expression in pET-20b. The first 10 N-terminal amino acids of mutant 12 A were confirmed by N-terminal protein sequencing.

Cultivation and purification f mutant and wild-type in pET-20b

In order to study the binding properties and stability of the mutant in detail, large amounts of mutant 12A and wild-type proteins were prepared. BL 21 cells were transformed with plasmids pET-20b/Mu 12A and pET-20b/Gamma-II-crystalline, respectively. The clones were cultivated by diluting a preculture 1:100 with LB medium/100µg/ml ampicillin and agitating the culture at 200 rpm and 37°C up to an OD_{600} of 0.5. Expression of the gamma-crystalline was induced by adding IPTG (final concentration 1 mM). Culturing was continued overnight at 30°C and 200 rpm. The bacteria cells were harvested by centrifugation at 4°C, 6 000rpm (Sorvall GS3 rotor) for 10 min. The cell pellet was suspended in 30 ml of 2 x PBS with addition of 150 µl of 200 mM PMSF and 10 µl of DNAse (Boehringer). The cells were then disrupted twice using a Gaulin press at 800-1000 PSIG. The supernatant containing the soluble proteins was obtained after centrifugation of the cell suspension at 4°C and 20 000 rpm (Sorvall SS 34 rotor) for 1 h. The gamma-crystallines fused to 6 histidine residues were purified by affinity chromatography at 4°C. 8 ml of Ni-NTA were equilibrated with 50 ml of 2 x PBS/10 mM imidazole. The supernatant containing the soluble proteins was then slowly agitated with the equilibrated column material in a bach process on a roller shaker overnight. Introducing the suspension into a chromatography column was followed by washing with 2 x PBS/10 mM imidazole/300 mM NaCl. The bound protein was eluted with 2 × PBS/ 250 mM imidazole. DTT (final concentration 10 mM) was added to the eluted proteins. This was followed by 2 dialysis steps at 4°C for in each case 8 h: 1st with 100 mM Na phosphate buffer pH 6.0/ 1 mM EDTA/1 mM DTT and 2nd with 10 mM Na phosphate buffer pH 6.0/ 1 mM EDTA. The supernatant obtained after a final centrifugation (4°C, 30 min, 20 000 rpm in Sorvall SS 34 rotor) contained the purified protein (Mu 12A or Gamma-II-crystalline) which was used for binding studies and stability studies.

Specific binding of mutant 12A to the BSA-estradiol-17-hemisuccinate conjugate was assayed by carrying out an ELISA, with increasing concentrations of purified mutant 12A-His-Tag protein being used. Increasing amounts of gamma-II-crystalline wild-type (likewise with His-Tag) were used as control, and binding of both purified proteins to BSA was assayed. The concentration-dependant ELISA was carried out using NUNC-Tm plates. Antigen coating with the BSA-estradiol-17-hemisuccinate conjugate or with BSA was carried out at room temperature overnight. Coating was carried out with in each case 100 µl of antigen at a concentration of 20 µg/ml of PBS pH 7.6. After washing (2 x PBS pH 7.6) and

blocking the plates (3% Marvel/PBS at 37°C for 2 h), in each case 1-13 µl of the protein stock solution (concentration 0.63 mg/ml) of purified Mu 12A or gamma-II-crystalline were introduced into a total 100 µl of reaction solution (PBS, 3% Marvel, x µl of protein) and incubated in the wells at 37°C for 2 h. The secondary antibodies used were the tetra-His antibody from Qiagen in a dilution of 1:3000 and the anti-mouse POD antibody (Sigma) in a dilution of 1:2000. The antibodies were diluted with a 3% strength Marvel/PBS solution and 100 µl were added to the wells and incubated at 37°C for in each case 1 h. The substrate reaction was carried out as described for the polyclonal phage ELISA. The result of this ELISA in Fig. 11 shows clearly that increasing extinctions are measured only with increasing concentrations of mutant 12A. No increase was detected using gamma-II-crystalline. Likewise, no reaction with BSA was observed. This shows specific binding of mutant 12A compared with the starting protein.

Stability was studied by recording guanidine denaturation because of mutant 12A and of gamma-II-crystalline. For this purpose, the purified proteins were incubated at a final concentration of 20 µg/ml with increasing concentrations of guanidinium at 20°C for one and three days. In total 15 guanidinium concentrations were adjusted in a range from 0 – 5.5 M in a 1 mM DTT/0.1 M Na phosphate buffer pH 6.0 solution. After one and three days, respectively, a 300-400 nm fluorescence emission spectrum of each mixture was recorded. The excitation wavelength was 280 nm. Fig. 12 depicts the emission maxima determined as a function of guanidinium concentrations. The stability of gamma-II-crystalline is higher than that of mutant 12A both after one day and after three days. However, compared with antibody molecules, the stability of mutant 12A is much higher.

Change in fluorescence properties of mutant 12A

Fluorescence spectra were recorded in order to test whether the fluorescence properties of mutant 12A have changed compared with wild-type protein. For this purpose, in each case 100 µg/ml of wild-type protein or mutant 12A (in 50 mM Na phosphate, pH 6.0) were excited at 280 nm and fluorescence was measured in a wavelength range from 300 to 400 nm in a cuvette of 1 cm pathlength. The slit width was 5 nm both for excitation and for emission.

The detected fluorescence signal had a maximum of 329 nm both for wild-type and for mutant 12A. However, the fluorescence intensity of mutant 12A, with only 86% signal intensity, was distinctly lower compared with gamma-crystalline wild-type (100%) (see Fig. 13A).

Mutant 12A and wild-type have an identical total number of fluorophores. However, sequence alterations in the mutant (Y -> K at Position 8 and C -> Y at Position 15) cause a change in the fluorescence signal. The difference in fluorescence intensity can be attributed to the fact that the tyrosine residues in positions 8 and 15, respectively, have different fluorescence properties.

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His His His His His
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DESIGN OF BETA-SHEET PROTEINS WITH SPECIFIC BINDING PROPERTIES

AMENDED CLAIMS

1. Protein with beta-sheet structure,
characterized in that
amino acids exposed on the surface in at least two β-strands exposed on the surface of at least one beta sheet exposed on the surface are specifically substituted, deleted or inserted, such that the protein has new specific antigen binding properties or a new catalytic activity or new fluorescence properties.

2. Protein according to Claim 1,
characterized in that
it is included in the group consisting of crystallines, spherulines, heat shock proteins, cold shock proteins, β-helix proteins, lipocalins, serpins, fibronectins or transcription factors or is GFP, NGF, tendamistat or lysozyme.

3. Protein according to Claim 1 or 2,
characterized in that,
amino acids exposed on the surface in three beta strands exposed on the surface are substituted, deleted or inserted.

4. Protein according to Claim 1 or 2,
characterized in that,
amino acids exposed on the surface in four or more beta strands exposed on

the surface are substituted, deleted or inserted.

5. Protein according one or more of the preceding claims,
characterized in that
amino acids exposed on the surface in at least two beta strands in at least two beta sheets are substituted, deleted or inserted.

6. Protein according to one or more of the preceding claims,
characterized in that,
amino acids exposed on the surface in three beta strands in two antiparallel beta sheets are substituted, deleted or inserted.

7. Protein according to one or more of the preceding claims,
characterized in that
it is a crystalline of vertebrates, preferably rodents, birds or fish.

8. Protein according to one or more of the preceding claims,
characterized in that,
it is an alpha-, beta- or gamma-crystalline.

9. Protein according to one or more of the preceding claims,
characterized in that,
it is a gamma-II-crystalline protein.

10. Protein according to one or more of the preceding claims,
characterized in that
amino acids exposed on the surface of the protein are substituted, deleted or inserted in a region of the beta sheet accessible to a solvent or to a binding partner.

11. Protein according to one or more of the preceding claims,
characterized in that,
amino acids exposed on the surface are substituted, deleted or inserted in a β-sheet structure of a domain or a subunit of the protein.

12. Protein according to one or more of the preceding claims,
characterized in that,
it is a gamma-II-crystalline which has been obtained by substitution, deletion or insertion of one or more of the amino acids Lys 2, Thr 4, Tyr 6, Cys 15, Glu 17, Ser 19, Arg 36 and Asp 38 in gamma-II-crystalline.

13. Protein according to one or more of the preceding claims,
characterized in that,
amino acids exposed on the surface of the protein have been substituted, deleted or inserted in the beta sheet such that it has antibody-like binding properties or an enzymic (catalytic) activity.

14. Protein according to Claim 12 or 13,
characterized in that
it has binding specificity for estradiol or the conjugate thereof, BSA-β-estradiol-17-hemisuccinate.

15. Protein according to one or more of the preceding claims,
characterized in that,
it has binding specificity for estradiol or the conjugate thereof, BSA-β-estradiol-17-hemisuccinate and has the amino acid sequence SEQ ID NO. 19 or SEQ ID NO. 21.

16. Protein according to one or more of the preceding claims,
characterized in that,
it is combined with other proteins or non-protein substances.

17. DNA coding for a protein according to one or more of the preceding claims.

18. RNA derived from the DNA according to Claim 17.

19. Prokaryotic or eukaryotic vectors or cells comprising a DNA or RNA according to Claim 17 or 18 or parts thereof coding for functional regions of the protein.

20. Method for preparing a protein according to one or more of the preceding claims, comprising the following steps:

a. Mutagenesis of the DNA coding for a protein with beta-sheet structure in those regions which code for at least two beta strands, exposed on the surface, of a beta sheet exposed on the surface;

b. Expression of the mutants obtained in step (a) in a suitable expression system; and

c. Selection and isolation of mutants having the desired binding properties and/or the desired catalytic activity; optionally

d. Expression and purification of the beta sheet-mutated proteins.

21. Method according to Claim 20,
characterized in that
the mutagenesis comprises a substitution, deletion or insertion of specific amino acid positions (site-specific mutagenesis) or non-specific amino acid positions (random mutagenesis) in the beta sheet.

22. Method according to one or more of the preceding claims,
characterized in that,
the mutants in step b) are expressed in prokaryotic or eukaryotic cells, in a cell-free system as a complex with ribosomes or on the surface of plant or animal cells, yeast cells or phages, viruses or bacteria.

23. Method according to one or more of the preceding claims,
characterized in that
mutants having the desired binding properties are selected by contacting these mutants with the binding partner and isolating those mutants having the desired binding affinity.

24. Method according to one or more of the preceding claims, characterized in that
mutants having the desired catalytic properties are selected by contacting these mutants with their substrate and isolating those mutants having the desired catalytic activity.

25. Use of a protein according to one or more of the preceding claims in diagnostics and therapy, in cosmetics, bioseparation and biosensors and reduction of harmful substances.

Application number/ Numéro de demande : EP00-06698

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Documents de pièrre qualité numérisés
(Pour obtenir les documents originaux, veuillez vous adresser à la Section de préparation des dossiers, située au 10e étage)

1/8

Fig. 1

GCLIE1B: Biotin-
CGCGCGCGTCTCACAAAGATACATGCCATGACTCGCGGCCCAGCC
GCLIE2P: P-
CCCCATGGCCGGCTGGGCCGCGAGTCATGGCATGTATCTTTGTGAGACGCGCGCG
GCLI3P: P- GGCCATGGGGNNKATCNNKTTTNNKGAGGACCGGGG
GCLIB4P: P- GTGGCCCTGGAAGCCCCGGTCCTC
GCLI5P: P-
CTTCCAGGGCCACNNKTACNNKTGCNNKAGCGACTGCCCCAACC
GCLI6P: P- TGCAGCCCTATTTCAGCCGC
GCLIB7P: P-
GATGGAGTTACAGCGGCTGAAATAGGGCTGCAGGTTGGGGCAGTCGC
GCLI8P: p-
TGTAACTCCATCNNKGTGNNKAGCGGCTGCTGGATGCTGTATGAG
GCLIE9P: P- CGCCCCAACTACCAGGGTCACCAGTACTTCCTGCGGC
GCLIE10:
GCCGCAGGAAGTACTGGTGACCCTGGTAGTTGGGGCGCTCATACAGCATC
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P: Phosphate, N: A/C/G/T, K: T/G

Fig. 2

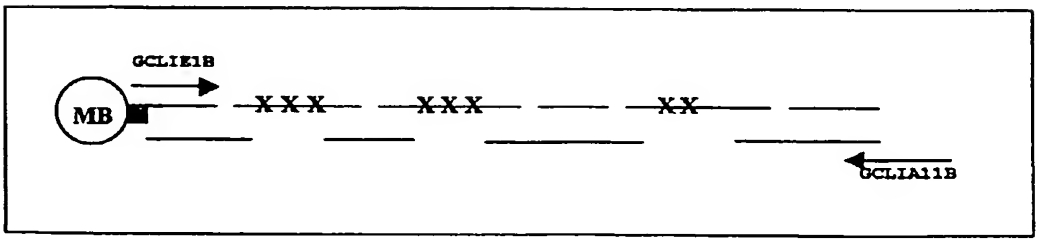

Fig. 3

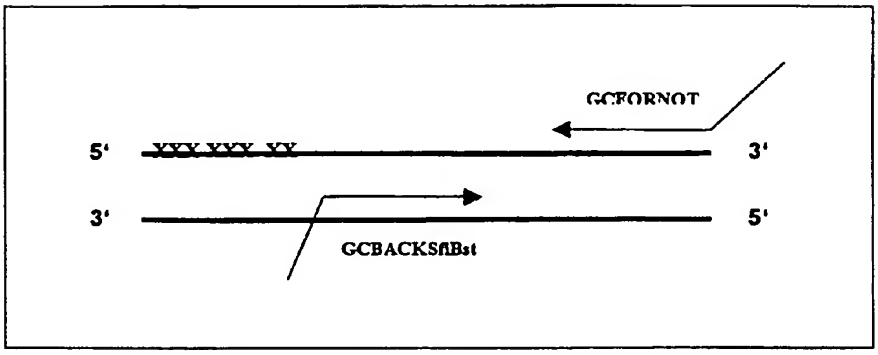

Fig. 4

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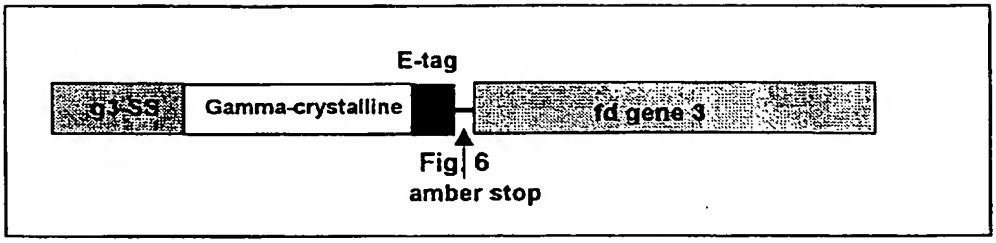
3/8

Fig. 5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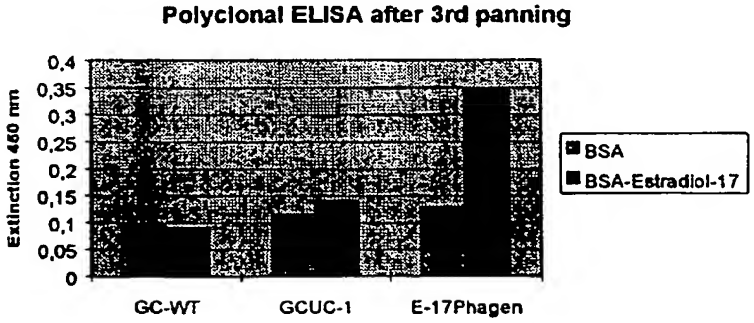
fd gene 3
Fig 6
amber stop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Extinction 450 nm
0,4
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
GC-WT
GCUC-1
E-17Phagen
BSA
BSA-Estradiol-17

Fig. 7

Mu 12A:
*GGCCCAGCCGGCC*ATGGGGAGGATCAAGTTTAAAGAGGACCGGGGGCTTCCAGGGCCA
CTATTACAGTTGCAATAGCGACTGCCCCAACCTGCAGCCCTATTTCAGCCGCTGTAACT
CCATCAGGGTGCTGAGCGGCTGCTGGATGCTGTATGAGCGCCCCAACTACCAG*GGTCA*
CC

WT:
*GGCCCAGCCGGCC*ATGGGGAAGATCACTTTTTACGAGGACCGGGGCTTCCAGGGCCA
CTGCTACGAGTGCAGCAGCGACTGCCCCAACCTGCAGCCCTATTTCAGCCGCTGTAAC
TCCATCCGCGTGGACAGCGGCTGCTGGATGCTGTATGAGCGCCCCAACTACCAGGGC
CACC

Fig. 8

Mu 12A:
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QYFLRRGDYPDYQQWMGFNDSIRSCRLIPQHTGTFRMRIYERDDFRGQMSEITDDCPSLQ
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FY*AAAGAPVPYPDPLEPRAA*

WT:
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FY*AAAGAPVPYPDPLEPRAA*

Fig. 9

GC 20bbackWT: 5' GGGAATTCCATATGGGGAAGATCACTTTTTACG 3'
GC 20bback12A: 5' GGGAATTCCATATGGGGAGGATCAAGTTTAAAG 3'
GC for 20b: 5' CGCGGATCCGAATAAAAATCCATCACCCG 3'

Fig. 10

Mu 12A-HIS:
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RGDYPDYQQWMGFNDSIRSCRLIPQHTGTFRMRIYERDDFRGQMSEITDDCPSLQDRFHL
TEVHSLNVLEGSWVLYEMPSYRGRQYLLRPGEYRRYLDWGAMNAKVGSLRRVMDFY*SDP*
NSSSVDKLAAALEHHHHHH

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RGDYPDYQQWMGFNDSIRSCRLIPQHTGTFRMRIYERDDFRGQMSEITDDCPSLQDRFHL
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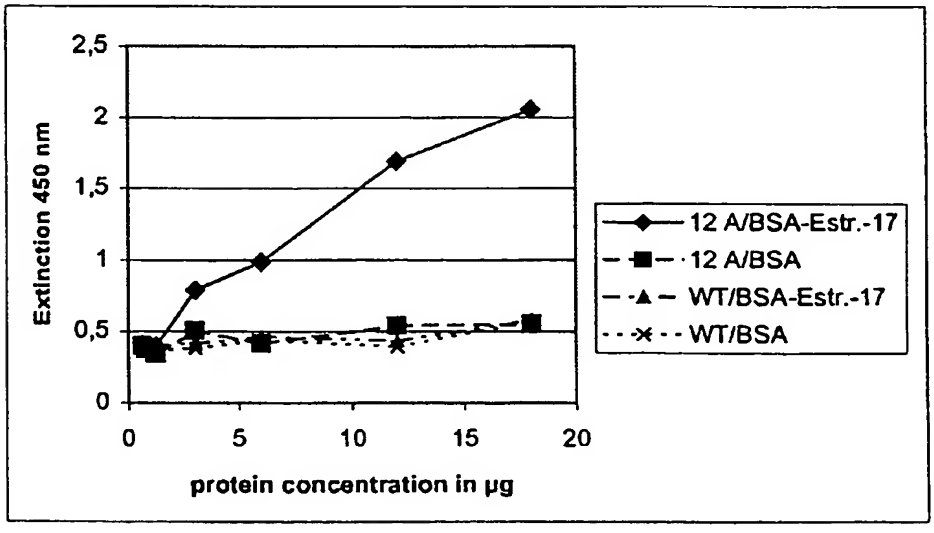
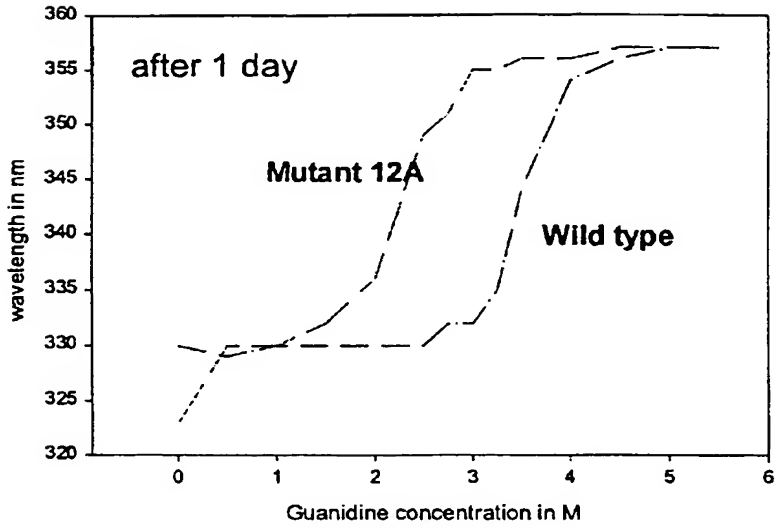
NSSSVDKLAAALEHHHHHH

Fig. 11



6/8

Fig. 12


after 1 day
Mutant 12A
Wild type
wavelength in nm
Guanidine concentration in M
360
355
350
345
340
335
330
325
320
0
1
2
3
4
5
6

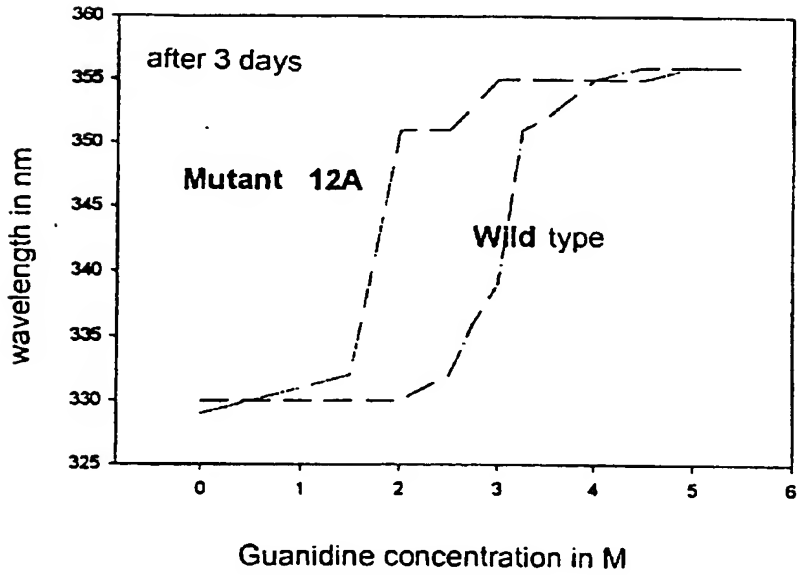

after 3 days
Mutant 12A
Wild type
wavelength in nm
Guanidine concentration in M
360
355
350
345
340
335
330
325
0
1
2
3
4
5
6

Fig. 13A

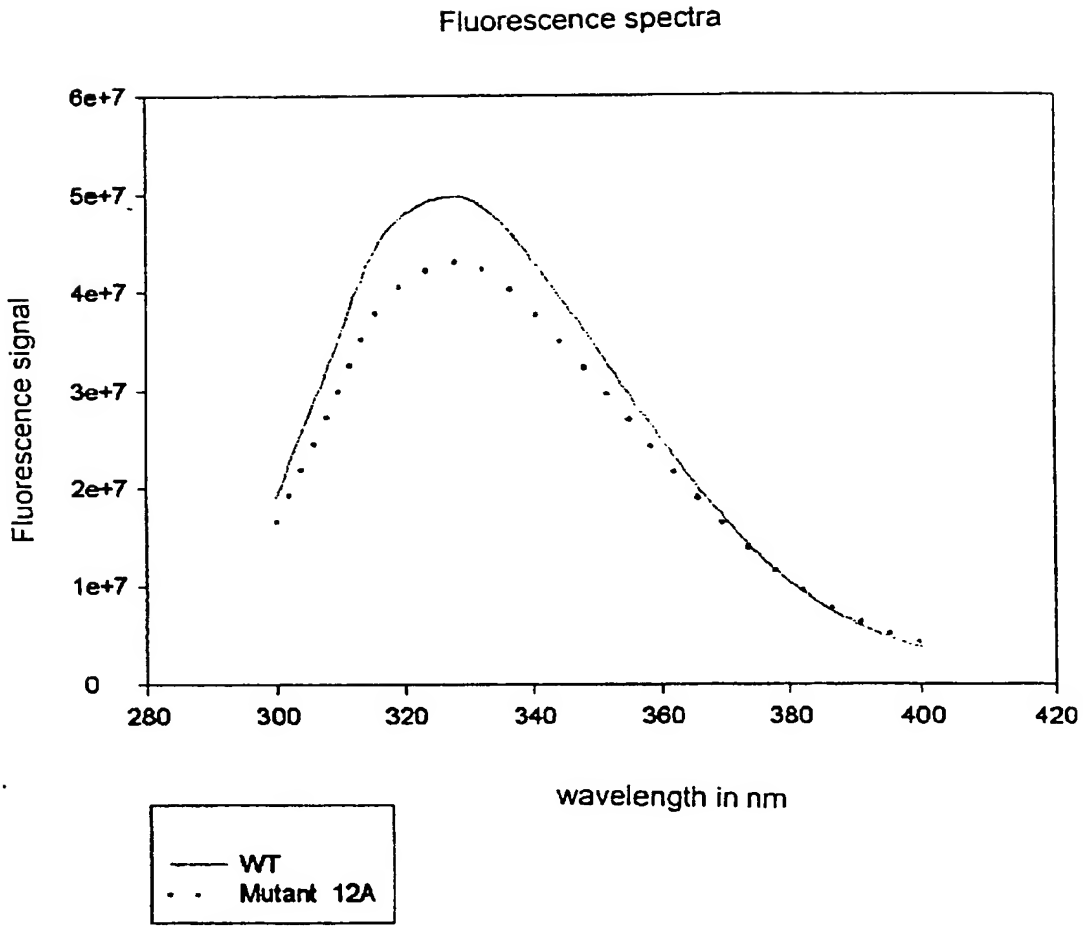
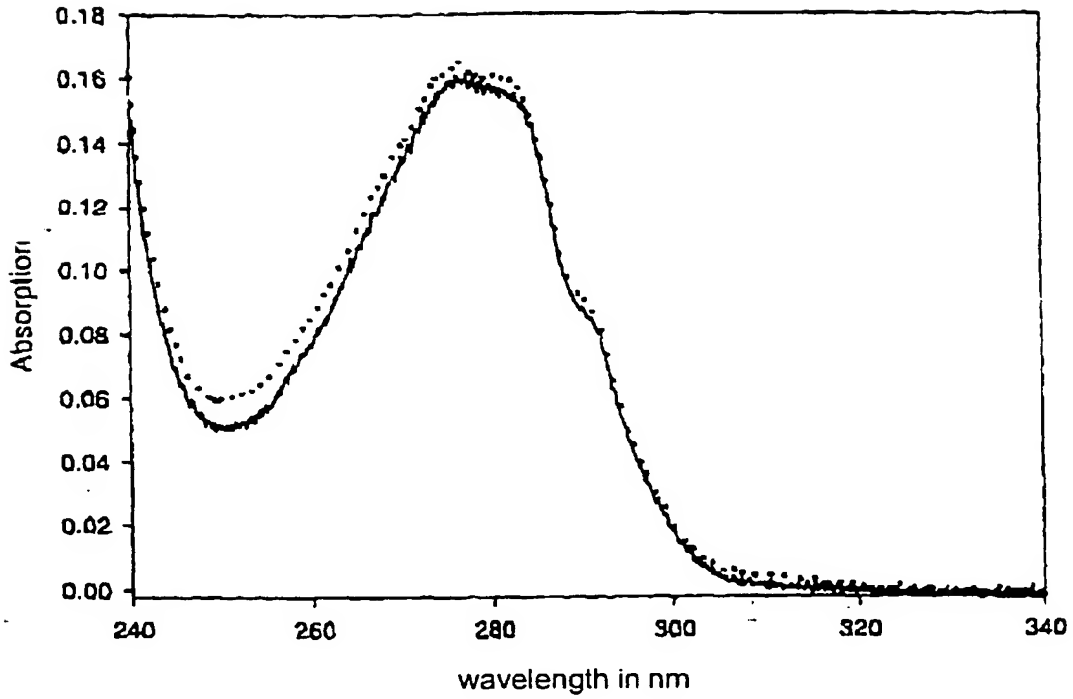

Fluorescence spectra
6e+7
5e+7
4e+7
3e+7
2e+7
1e+7
0
Fluorescence signal
280
300
320
340
360
380
400
420
wavelength in nm
WT
Mutant 12A

Fig. 13B


0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorption
240
260
280
300
320
340
wavelength in nm